Analysis of the Prodrug 7-Acetylacroninium Perchlorate in Presence of the Parent Compound Acronine

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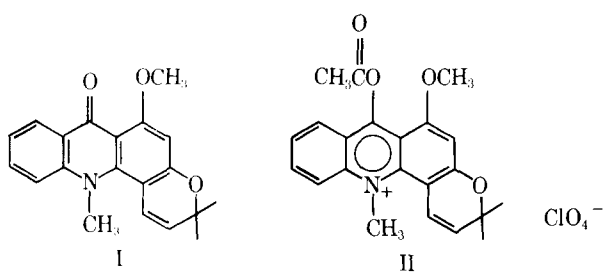
Abstract □ Analysis of acronine and the prodrug acetylacroninium perchlorate in parenteral solutions is described. The unstable prodrug reacts quantitatively with the nucleophilic agent aniline by an unusual mechanism to form a phenylimino derivative of acronine. This derivative and acronine itself were determined by a spectrophotometric two-component method.

Keyphrases □ 7-Acetylacroninium perchlorate—two-component spectrophotometric analysis with acronine in parenteral solutions □ Acronine—two-component spectrophotometric analysis with 7-acetylacroninium perchlorate in parenteral solutions □ Spectrophotometry—two-component analysis, acronine and 7-acetylacroninium perchlorate in parenteral solutions □ Prodrugs—7-acetylacroninium perchlorate, two-component spectrophotometric analysis with acronine in parenteral solutions □ Antineoplastic agents—acronine, two-component spectrophotometric analysis with 7-acetylacroninium perchlorate in parenteral solutions

Clinical evaluation of parenteral administration of the water-insoluble cytotoxic agent acronine[1] (I) (1, 2) resulted in the preparation of the prodrug acetylacroninium perchlorate (II) (3). The formulation of a suitable parenteral solution of the prodrug was described previously (4).

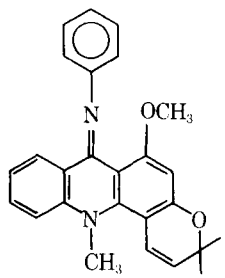
Since it has not been possible to prepare samples of acetylacroninium perchlorate free from unreacted acronine perchlorate (3) and since the prodrug is not stable ($t_{1/2} \sim$ 25 min at 25° in water), problems were encountered in analysis of the prodrug. In a previous report, a method was described (4) by which the prodrug was extracted into chloroform, presumably as an ion-pair with gentisate ion, and the absorptivity was measured at 520 nm. The molar absorptivity of this ion-pair was estimated by an indirect method (4).

This report deals with a direct measurement of the ester and parent compound by utilizing the ester's high reactivity with the nucleophile aniline. The reaction product (III), which is essentially the Schiff base of acronine and aniline, and acronine itself were determined by two-component visible spectrophotometry. The method is compared with the previously published methods.



I II

[1] NSC 403169; referred to as acronycine in previous publications.



III

EXPERIMENTAL

Materials—Acronine was used as obtained[2]. The purity of the sample was examined by a phase solubility analysis procedure similar to the general method described by Mader (5). The solvent was methanol–water (3:1), and the sample was equilibrated for 44 hr at 25.0 ± 0.1° and protected from light.

Calculations based on the solubility diagram (5) showed a content of 100.5% acronine in the sample, indicating no significant amounts of impurities. This result was in agreement with the fact that TLC experiments revealed no secondary spots (<0.5%) with detection by iodine vapor or UV light at 254 nm. Acetylacroninium perchlorate was prepared as described by Bourne *et al.* (3).

The chloroform was purified by distillation. Aniline was purified by distillation *in vacuo* and stored under nitrogen in a refrigerator. All other chemicals were reagent grade and were used without further purification. The TLC plates were 0.25-mm silica gel 60 F_{254} precoated plates[3]. The mobile phase in TLC was ethyl acetate–cyclohexane–25% ammonia (40:10:1). Silica gel[4], 0.05–0.2 mm, was used for column chromatography.

Synthesis and Characterization of III—Preliminary experiments showed that the same reaction between the prodrug and aniline took place (*i.e.*, the same reaction product was formed and to the same extent) in aqueous solution and in organic solvents. Compound III was prepared by dissolving 680 mg of II in 50 ml of chloroform and then adding 1.0 ml of aniline. The reaction was complete within a few seconds, and the chloroform solution was extracted with 50 ml of 1 *N* HCl to remove excess aniline quantitatively.

Conversion of the salt of III into the corresponding base, which is more readily chromatographed, was accomplished by shaking the chloroform solution with 50 ml of 2 *M* ammonia. Residual water in the organic layer was removed by filtration through anhydrous sodium sulfate, and the chloroform was removed under reduced pressure at 30°. The residue was crystallized from water–methanol to yield a yellow product (580 mg), a mixture of I and III.

Isolation of III was by column chromatography on 80 g of silica gel. The column (2 cm i.d.) was eluted by cyclohexane–ethyl acetate–25% ammonia (50:50:1), the fractions containing III were collected, and the organic solvent was removed under reduced pressure. The product thus obtained was recrystallized twice from hot methanol and dried *in vacuo* at 40° for 18 hr (yield of 410 mg).

The suggested structure of the compound, mp 219–220° (uncorrected), obtained from the reaction between II and aniline is shown as III. The elemental analysis[5] for carbon, hydrogen, and nitrogen of III (mol.

[2] National Cancer Institute, Bethesda, Md.
[3] Merck No. 5715.
[4] Merck No. 7734.
[5] Performed by Microchemical Laboratory, University of Copenhagen.

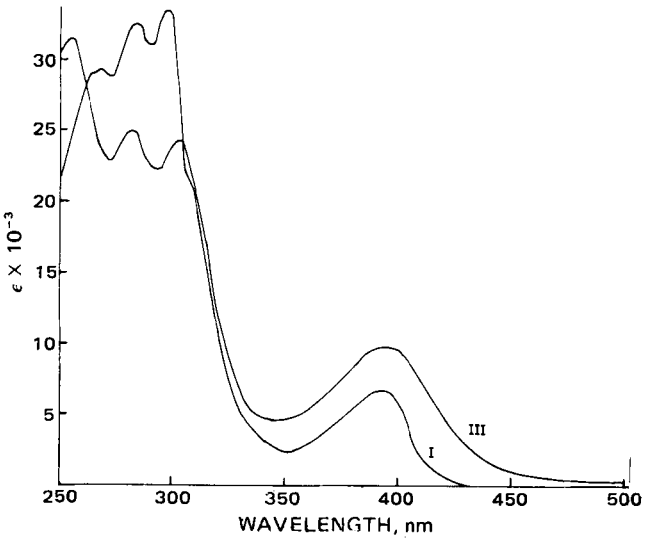


Figure 1—*Absorption spectra in chloroform of I and the product (III) formed by reaction between II and aniline.*

wt. 396.5) was ±0.3% of the calculated figures. The NMR spectrum of III was similar to that of I (3) with the exception of five aromatic protons at δ 6.8–7.4 ppm (multiplet). The mass spectrum supports the structure of III with a parent ion of *m/e* 396 (27%) and a base peak of *m/e* 395 ($M^+ - 1$).

The acid dissociation constant, pKa, for protonated III was determined at 25° and an ionic strength of 0.05 by the spectrophotometric method described by Albert and Sergeant (6). Due to the very low solubility of III in water, 10% methanol was added to the buffer solutions. The analytical wavelength was 292 nm, and 1.00-cm cells were used. The pKa, 8.7, is of an order of magnitude expected for compounds with a protonated imino nitrogen (7).

Preparation of Samples for Analysis—A solution of sodium gentisate (0.8 *M*, pH 6) was prepared by mixing the necessary quantity of gentisic acid, 10% sodium hydroxide, and water (4). To 400 ml of the gentisate solution was added 950 mg of II. The sample was placed in an ultrasonic bath for 10 min, removed, and vigorously agitated for an additional 10 min. The resulting clear solution was frozen in sealed ampuls and stored at −40° in an air thermostat. The samples were kept for 0.5–3 hr at room temperature prior to analysis.

Two samples (c and d) were prepared by extracting most of I from the sample described above with ether, removing the ether under reduced pressure, and shaking the sample thus obtained with excess of solid II (containing I as an impurity). These mixtures were finally filtered to obtain a clear solution.

Analytical Procedure—*Method A: Aniline Reaction, Two-Component Spectrophotometry*—A solution containing an aliquot (2.00 ml) of the test solution, water (7.00 ml), and aniline (0.1 ml) was extracted with three 7.0-ml portions of chloroform. The combined chloroform extracts were shaken with 1 *N* HCl (15.0 ml) to remove excess aniline. Removal of aniline was necessary to avoid the possible interference of colored products formed by oxidation of aniline.

Finally, the chloroform extracts were shaken with borate buffer, pH 10.5 (20 ml), filtered through anhydrous sodium sulfate, and diluted with chloroform to 25 ml. The absorbance of the solution was measured at 440 nm and, after a fivefold dilution, at 393 nm. Calculations of the content of I and III were done according to general equations for two-component systems (8).

Method B: Spectrophotometry of 7-Acetylacroninium Ion (II)—The intact prodrug was analyzed as previously described (4) with the following minor modification. It was found that the decrease of A_{520} in chloroform solutions of the prodrug ion proceeded with a variable rate and that this decrease followed pseudo-zero-order kinetics. Therefore, the value of A_{520} for the chloroform extract was read at ~10-min intervals for ~40 min after the first reading and extrapolated to the time of initiation of the extraction. Since it was not possible to prepare a pure sample of II and since II is extremely unstable in aqueous and nonaqueous solvents, it was not possible to determine the accuracy or precision of the method.

Method C: Spectrophotometry of Total Acronine (I)—The total content of free and esterified acronine was analyzed after complete hydrolysis of the prodrug as described previously (4). The blank solution was prepared from a 0.8 *M* solution of sodium gentisate treated as the test solution. The relative standard deviation of the method was 0.9% ($n = 10$).

Table I—Analytical Results for II (Calculated as I) and I in Various Parenteral Solutions[a]

Sample	Compound	Method A	Method B	Method C
a	II	77.2	69.2	—
	I	25.1	—	—
	I + II	102.3	—	100 (1.67)
b	II	67.7	64.8	—
	I	35.9	—	—
	I + II	103.6	—	100 (1.67)
c	II	52.2	49.0	—
	I	44.3	—	—
	I + II	96.5	—	100 (2.52)
d	II	39.5	39.8	—
	I	53.8	—	—
	I + II	93.3	—	100 (2.53)
e	II	72.8	69.6	—
	I	29.9	—	—
	I + II	102.7	—	100 (1.66)
f	II	58.8	58.1	—
	I	41.9	—	—
	I + II	100.7	—	100 (1.68)
g	II	73.3	71.9	—
	I	27.9	—	—
	I + II	101.2	—	100 (1.67)
h	II	61.8	58.5	—
	I	37.6	—	—
	I + II	99.4	—	100 (1.64)

[a] The values stated are expressed in percentage of the total concentration of free and esterified I determined by Method C and are the mean of a duplicate analysis. The values in parentheses are the actual values of I plus II in milligrams of I per milliliter determined using Method C.

RESULTS

***In Situ* TLC Qualitative Studies on Aniline Reaction**—Qualitative studies on the reaction between II and aniline were performed using *in situ* reflectance measurements of TLC plates as described elsewhere (9). With the described TLC system, acetanilide, a possible reaction product between aniline and 7-acetylacroninium perchlorate (II), was well separated from I and III. Experiments using 242 nm (reflectance minimum of acetanilide) as the analytical wavelength showed that acetanilide was not formed (detection limit ~1% of II) in a sample of II to which excess aniline was added. The only reaction product detected was III (range of analytical wavelength was from 220 to 400 nm with 10-nm increments). Furthermore, aniline did not react with I.

Two-Component Spectrophotometry—The absorption spectra of I and III are shown in Figure 1. The wavelengths for analysis were 393 nm, where both I and III showed maximum absorbance, and 440 nm, where the absorbance of I was small compared to that of III. The molar absorptivities determined from Beer law-type plots were $\epsilon_{393} = 6700\ M^{-1}\ cm^{-1}$ [this value was previously reported to be $6950\ M^{-1}\ cm^{-1}$ (4)] and $\epsilon_{440} \sim 15\ M^{-1}\ cm^{-1}$ for I and $\epsilon_{393} = 9910\ M^{-1}\ cm^{-1}$ and $\epsilon_{440} = 1710\ M^{-1}\ cm^{-1}$ for III.

To examine the precision of the method, nine determinations were made on a test solution containing approximately 1.5 mg of 7-acetylacroninium perchlorate and 0.5 mg of acronine/ml. The relative standard deviations were 1.0 and 4.3%, respectively.

Comparison of Three Methods—Table I shows the results of analysis, using the described methods, of eight samples containing unknown amounts of I and II (see *Experimental*).

DISCUSSION

A possible reaction between aniline and the acetyl ester (II) might be expected to be the aminolysis of II with the formation of acetanilide (10). However, acetanilide was not formed, and III was the only reaction product found. The mechanism for the formation of III from aniline and II appears to involve direct and rapid attack of the nucleophile at the 7-carbon and displacement of the acyl group. This mechanism may be rationalized on the basis of the electrodeficient character of the nitrogen and the resonance between the quaternary nitrogen and the carbon in the 7-position. This carbon is electropositive, and a nucleophilic attack at C-7 is possible. This is in agreement with the fact that aryl oxygen

cleavage was shown (11) to be a significant reaction in the hydrolysis of II in water.

Samples were analyzed using the three methods described (Table I). The total of I plus II calculated as I determined using Method A agrees with the total content of I determined after complete hydrolysis. This fact, together with the observation that III is the only reaction product formed from II and aniline, strongly indicates that Method A provides an accurate measurement of I and II. With the exception of Sample a, only small deviations can be observed between the content of II determined by Methods A and B. This result may indicate that the value of $\epsilon_{520} = 6200$ M^{-1} cm^{-1} for II (4) is not correct and that using a value of $\epsilon_{520} = 6020$ M^{-1} cm^{-1} leads to improved agreement between the results of Methods A and B.

The methods described are intended to be applied to II and I in a 0.8 *M* solution of sodium gentisate (4). The present work shows that the reaction between II and aniline is applicable for the determination of a mixture of I and II in parenteral solutions and that spectrophotometric analysis of the reaction mixture appears to be a simple and reliable method of analysis.

REFERENCES

(1) G. H. Svoboda, G. A. Poore, P. J. Simpson, and G. B. Boder, *J. Pharm. Sci.*, **55**, 758 (1966).

(2) G. H. Svoboda, M. J. Sweeney, and W. D. Walkling, *ibid.*, **60**, 333 (1971).

(3) D. W. A. Bourne, T. Higuchi, and A. J. Repta, *ibid.*, **66**, 628 (1977).

(4) C.-H. Huang, B. Kreilgård, and A. J. Repta, *Bull. Parenteral Drug Assoc.*, **30**, 1 (1976).

(5) W. J. Mader, *CRC Crit. Rev. Anal. Chem.*, **1**, 193 (1970).

(6) A. Albert and E. P. Sergeant, "Ionization Constants of Acids and Bases," Methuen, London, England, 1962.

(7) A. C. Capomacchia, J. Casper, and S. G. Schulman, *J. Pharm. Sci.*, **63**, 1272 (1974).

(8) H. H. Willard, L. L. Merritt, Jr., and J. A. Dean, "Instrumental Methods of Analysis," 5th ed., Van Nostrand, New York, N.Y., 1974, p. 92.

(9) A. B. Hansen and S. S. Larsen, *Dan. Tidsskr. Farm.*, **46**, 105 (1972).

(10) K. A. Conners, "Reaction Mechanisms in Organic Analytical Chemistry," Wiley, New York, N.Y., 1973, p. 516 ff.

(11) A. J. Repta, J. R. Dimmock, B. Kreilgård, and J. J. Kaminski, *J. Pharm. Sci.*, **66**, 1501 (1977).

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Comparison of Solid and Solution Conformations of Hydroxyurea and 3-Ethyl-1-hydroxyurea Utilizing IR–X-Ray Method

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Abstract □ The IR–X-ray method for determining solution conformation was used to compare the conformations of the antitumor agents hydroxyurea and its 3-ethyl analog in solutions with conformations in the solid state. X-ray crystal analysis data for hydroxyurea obtained from the literature and the 3-ethyl analog data determined experimentally indicated that similar conformations are present in both compounds, with a carbonyl bond of mostly double bond character and intermolecular hydrogen bonding. The ethyl analog also possessed inter- and intramolecular hydrogen bonding involving the 1- and 3-hydrogens and the carbonyl and hydroxyl oxygens. Comparison of IR spectra (1780–1550 cm^{-1}) of the solid state (mineral oil mulls) with solution spectra (taken in dry octanol, in octanol equilibrated with water and dried, and in wet and dry acetonitrile) indicated that only in dry acetonitrile did the spectrum of hydroxyurea resemble that of the solid. The ethyl analog possessed absorptions similar to the solid only in dry octanol, while spectra of all other solutions for both compounds had strong C=N character and diminished C=O double bond character. The IR solution data were rationalized according to the conformational system of hydroxyureas, which is represented by canonical forms possessing a C=O bond or strong C=N bonds and a CO bond. Hydroxyurea appears to prefer hydrogen bonding to a solvent (water) to bonding with itself, while the ethyl analog possesses internal hydrogen bonding which changes the conformational preferences and conformational equilibrium. In solvents where this inter- or intramolecular hydrogen bonding competes with bonding with the solvent, interconversion of conformers takes place; but this interconversion cannot occur when strong hydrogen bonding (with solvate formation) exists with water. Hydroxyurea tends to exhibit this behavior only when hydrogen bonding to solvent or water is not possible, allowing interconversion through a form possessing a C=O bond. Hydroxyurea and its 3-ethyl analog thus have different solution conformational equilibria depending on the solvent.

Keyphrases □ Hydroxyurea and 3-ethyl analog—comparison of solid and solution conformations □ Conformations—solid and solution compared, hydroxyurea and 3-ethyl analog □ Antineoplastic agents—hydroxyurea and 3-ethyl analog, comparison of solid and solution conformations

The structure or conformation of drugs in solution is a significant property to scientists studying the molecular dynamics involved in absorption, *in vivo* transport, site of action, and enzymatic biotransformations of biologically active compounds (1). Knowledge concerning the actual or potential structure or conformation of drug molecules becomes even more relevant to the molecular pharmacologist or medicinal chemist if a particular structure or conformation is essential for a biological process and that particular molecular species is one of many possible species that may be present *in vivo*.

While the structure of molecules in the solid state can be ascertained with a high degree of accuracy *via* X-ray crystallography, the structure and conformation of molecules in simple solutions are continually being studied and are topics of rigorous debate (2). Various methods have